

ADENOVIRUS RECOMBINANTS DEFECTIFS EXPRIMANT
DES CYTOKINES POUR TRAITEMENT ANTITUMORAL

Les cytokines sont des molécules (hormones) produites par des cellules à la suite d'une stimulation antigénique ou d'une activation par d'autres facteurs. La première cytokine qui ait été produite est l'interleukine 1 (IL-1). Elle permet l'activation des cellules T, qui se mettent à leur tour à produire toute une batterie de lymphokines dont certaines sont indispensables pour l'activation du système immunitaire et les défenses contre les infections virales ou parasitaires.

Depuis quelques années, les cytokines sont utilisées en immunothérapie anticancéreuse. Néanmoins leur administration par la voie générale pose un certain nombre de problèmes. L'IL-2 par exemple donne des effets secondaires assez importants; elle est rapidement métabolisée, de sorte que de fortes doses doivent être administrées de façon répétée.

On est donc à la recherche de meilleures voies d'administration qui augmenteraient leur efficacité, tout en diminuant leurs effets indésirables.

L'invention a donc pour objet des adénovirus recombinants défectifs exprimant une ou plusieurs cytokines, ainsi que l'utilisation de ces adénovirus recombinants pour la constitution de compositions pharmaceutiques, notamment antitumorales, plus particulièrement de compositions directement injectables dans des tumeurs solides de l'hôte.

La présente invention a pour objet des adénovirus recombinants défectifs caractérisés en ce qu'ils comportent un génome d'adénovirus non répliquable, défectif dans lequel sont insérées une ou plusieurs séquences d'acide nucléique codant pour une ou

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plusieurs cytokines, notamment lymphokines, sous le contrôle d'un ou plusieurs promoteurs susceptibles d'être reconnus par les polymérases de cellules humaines, plus particulièrement de cellules tumorales humaines ou de cellules infiltrant es tumeurs.

L'invention concerne plus particulièrement les acides nucléiques recombinants susceptibles d'être mis en oeuvre pour la production de tels adénovirus recombinants défectifs.

Un tel acide nucléique recombinant est caractérisé en ce qu'il comporte, d'une part, une séquence génomique d'un adénovirus défectif en ce qu'elle est dépourvue des séquences nécessaires à sa réplication, mais comportant néanmoins celles des séquences qui dans ce génome sont le support de l'information génétique nécessaire à l'adénovirus correspondant pour pénétrer dans les cellules infectables par celui-ci, ainsi que l'ensemble des séquences essentielles nécessaires à l'encapsidation de cet adénovirus, et, d'autre part, un insérat contenant une séquence nucléique codant pour une cytokine, cet insérat étant sous le contrôle d'un promoteur présent ou préalablement inséré dans la susdite séquence génomique.

Les adénovirus, notamment les adénovirus de type 2 ou 5 susceptibles d'infecter les humains (ou adénovirus humains), ou encore les adénovirus de sérotype 4 et 7, représentent des vecteurs particulièrement préférés dans le cadre de la présente invention, en raison notamment de la grande taille du fragment d'ADN étranger qu'il est possible d'insérer dans le génome de ces virus.

Avantageusement, la ou les séquence(s) d'acide nucléique d'insertion sus-mentionnée(s), codant pour une ou plusieurs cytokines prédéterminées, sont contenues dans un génome défectif d'adénovirus,

dépourvu des séquences nucléotidiques essentielles nécessaires à la réplication de ces adénovirus, et plus particulièrement des transactivateurs E1A et E1B et, le cas échéant, de la région E3 de l'adénovirus, ou encore de ses régions E1 et E3.

En d'autres termes l'invention met à profit la capacité de ces adénovirus recombinants défectifs d'autoriser l'expression de la séquence d'insertion qu'ils contiennent dans les cellules qu'ils envahissent, même lorsqu'en raison de leur caractère défectif ils ne s'y multiplient pas. En d'autres termes l'invention a pour objectif de faire sécréter les cytokines au sein même des cellules de la tumeur à traiter (cellules tumorales elles-mêmes et cellules, notamment lymphocytes, qui infiltrent ces tumeurs) lorsque celles-ci ont été infectées par ces adénovirus défectifs, en particulier lorsque ceux-ci sont injectés directement dans la tumeur. Les cytokines produites activeront ainsi en priorité, in situ, les cellules cytotoxiques infiltrant la tumeur et celles se trouvant à proximité de la tumeur.

En ce qui concerne la séquence d'insertion dans le génome d'adénovirus recombinant défectif, elle peut être choisie parmi toutes celles qui expriment une cytokine capable d'exercer un effet, soit antitumoral direct, soit activateur de cellules immunocompétentes de l'organisme, soit les deux à la fois.

Parmi ces cytokines on mentionnera à titre d'exemples : IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 interféron α , interféron γ , "tumor necrosis factor" alpha (TNF α) (facteur alpha de nécrose de tumeur).

Les mêmes adénovirus recombinants peuvent aussi être utilisés dans le cas de certaines maladies, où il y a une déficience immunitaire et dans le cas de certaines maladies parasitaires ou virales (en particulier interféron γ , α et/ou GM-CSF), notamment

par administration par la voie générale ou par l'intermédiaire de cellules, de préférence humaines prises dans un état autorisant leur injection chez l'homme, ces cellules ayant au préalable été infectées par un adénovirus défectif recombinant conforme à l'invention.

On rappelle ci-après les propriétés de certaines de ces cytokines.

Interleukine 1 (IL-1) :

Elle est produite essentiellement par les monocytes et les macrophages activés. Son poids moléculaire est d'environ 17 Kilodaltons. Elle présente plusieurs activités, parmi lesquelles :

a) une action chemoattractive sur les cellules polymorphonuclées et les macrophages (1,2),

b) une augmentation de l'activité cytotoxique des cellules cytotoxiques spontanées ("Natural Killer", ou Nk),

c) une induction de fièvre à la suite d'une infection,

d) surtout l'activation des cellules T pour la production d'autres facteurs.

Interleukine 2 (IL-2), Interleukine 4 (IL-4) et Interleukine 5 (IL-5) :

Elles sont produites par des lymphocytes T activés. L'action de ces cytokines est restreinte aux cellules du système immunitaire, elles provoquent leur multiplication et leur activation : IL-2 et IL-4 ont été essayés en immunothérapie antitumorale chez la souris et chez l'homme. Chez la souris, elles agissent de façon synergique et provoquent la régression tumorale.

Interleukine 6 (IL-6)

Elle est produite par des nombreux types cellulaires dont les lymphocytes T, les macrophages, les fibroblastes... Elle induit la différenciation

finale des lymphocytes B qui deviennent producteurs d'anticorps.

"Tumor Necrosis Factor- α " (TNF- α) (facteur- α de nécrose des tumeurs) :

C'est un facteur produit par les macrophages. Il a une double action : une action directe sur les cellules tumorales en provoquant leur lyse et une activation du système immunitaire.

L'utilisation du TNF- α chez l'homme doit se faire avec précaution, étant donné que de nombreuses cellules en possèdent le récepteur : d'où l'intérêt de n'induire sa sécrétion que localement, au sein même de la tumeur, pour limiter ses effets non désirables sur les autres cellules de l'hôte.

Interleukine 3 (IL-3), Interleukine 7 (IL-7), et "Colony stimulating Factor" (CSF).

Ce sont des facteurs de croissance hématopoiétique. Ils sont produits essentiellement par les lymphocytes, les monocytes et les macrophages. Ils agissent à différents niveaux de l'hématopoïèse, c'est-à-dire des différentes étapes de différenciation de cellules de moëlle en cellules sanguines. En outre le CSF exerce des effets très importants au niveau des défenses primaires de l'organisme vis-à-vis des défenses bactériennes en attirant les macrophages vers les sites d'infection et en augmentant leur capacité de phagocytose.

En combinaison avec l'IL-2 et l'IL-4, le GM-CSF, s'avère être un important facteur antitumoral.

L'interféron γ (IFN γ)

C'est un facteur produit par les cellules T activées; il est doué de propriétés antivirales; il inhibe la multiplication des virus et des parasites et provoque la lyse des cellules infectées et de certaines cellules tumorales.

L'interféron α (IFN α)

Produit par des cellules T et des monocytes, il présente un effet antiviral et lytique sur des cellules infectées. L'IFN α a été utilisé en immunothérapie contre certains types de cancer dont le mésothélium.

Bien entendu l'invention n'est pas limitée, quant au choix des séquences d'insertion utilisables dans des adénovirus conformes à l'invention, à celles qui ont été identifiées ci-dessus. Néanmoins celles-ci sont illustratives de la palette des possibilités qui s'offrent au thérapeute, à qui appartient le choix de l'adénovirus recombinant défectif le plus approprié à mettre en oeuvre, compte tenu de la nature des tumeurs à combattre.

L'invention concerne également des compositions pharmaceutiques comprenant un ou plusieurs vecteurs recombinants tels que décrits ci-dessus, en association avec un véhicule pharmaceutiquement acceptable, en particulier des compositions directement injectables dans les tumeurs à traiter, stériles, isotoniques, ou des compositions sèches, notamment lyophilisées, qui, par addition selon le cas d'eau stérilisée ou de sérum physiologique, permettent la constitution ou reconstitution de solutés directement injectables dans les tumeurs.

L'injection directe dans la tumeur d'adénovirus modifiés, non répliquables, offre l'avantage d'une part d'éviter la diffusion des adénovirus recombinants dans la circulation générale avec, pour conséquence, les effets secondaires susceptibles d'être exercés par les cytokines exprimées ailleurs que sur les sites où la manifestation de leur action est recherchée, en l'occurrence les cellules tumorales, elles-mêmes ou les cellules, notamment lymphocytes, qui les infiltreront ou qui se trouvent à leur proximité immédiate. De

préférence, l'injection est réalisée à tout le moins dans au moins un site de la tumeur primaire.

L'invention n'est pas davantage limitée à l'administration des adénovirus recombinants contenant les séquences codant pour les cytokines du genre en question directement dans les tumeurs. Toute autre voie d'administration permettant un accès de ces adénovirus recombinants à la tumeur à traiter peut être envisagée. En particulier, on peut avoir recours à des cellules compatibles avec l'hôte, par exemple des fibroblastes humains, de préférence préalablement prélevés chez l'hôte lui-même.

L'invention concerne également des cultures cellulaires, par exemple des fibroblastes en culture préalablement infectés par des acides nucléiques recombinants, plus particulièrement les adénovirus défectifs sus-définis. Ces cellules infectées, le cas échéant atténuées ou rendues immunologiquement inertes, par exemple par irradiation, contribuent à l'éradication de tumeurs installées, lors de leur injection par la voie générale. Cette injection peut être envisagée soit seule, soit en sus de l'injection directement dans la tumeur.

L'invention a également pour objet un procédé d'obtention des adénovirus recombinants décrits ci-dessus qui comprend, après l'étape de construction proprement dite d'un vecteur par introduction d'un ou plusieurs acide(s) nucléique(s) d'insertion dans le génome de l'adénovirus défectif initial, une étape de transformation de lignées cellulaires transformables d'eucaryotes supérieurs (notamment d'origine humaine ou animale) comportant elles-mêmes une séquence distincte de nucléotides apte à compléter la ou les parties qui font défaut au génome de l'adénovirus défectif et sans lesquelles la répllication de ce dernier est interdite, ladite séquence distincte étant

de préférence incorporée au génome des cellules de ladite lignée cellulaire.

A titre d'exemple préféré de telles lignées cellulaires, on mentionnera la lignée 293, lignée de rein embryonnaire humain qui contient, intégrés dans son génome, les onze premiers pourcents de l'extrémité gauche du génome d'un adénovirus de type 5 (Ad5). Ceux-ci peuvent alors compléter des virus recombinants défectifs qui portent des délétions de cette région. Un tel procédé d'obtention est plus particulièrement décrit dans la demande de brevet européen n° 0 185 573 du 20/11/85.

Après transformation de ces lignées cellulaires, les adénovirus défectifs ainsi multipliés et produits sont récupérés à partir du milieu de culture des cellules de ces lignées et purifiés.

La présente invention sera d'avantage détaillée dans la description qui suit des possibilités de construction d'un adénovirus vecteur recombinant contenant au moins une séquence codant pour une cytokine, en particulier une lymphokine.

I. METHODES

a) Cellules et virus

La lignée cellulaire 293 rénale embryonnaire humaine transformée par Ad-5 (Graham et al., 1977), a été utilisée pour la transfection d'ADN ainsi que pour la multiplication et la titration d'Adénovirus (Ad). En effet, la lignée cellulaire 293 complète les fonctions des gènes de fonctions E1A et E1B et permet la répllication des Ad-recombinants défectifs. Pour la construction de l'Ad recombinant, l'Ad5-dl324 humain, portant des délétions dans les régions E1 (3.9-10.5 m.u.) et E3 (78.5-84.3 m.u.), a été utilisé (Shenk et Williams, 1984). Les lignées cellulaires 293, Hela et

Vero ont été maintenues dans un milieu de culture minimum essentiel Eagle avec 10 % de sérum de veau fœtal.

b) Construction des plasmides permettant l'expression de différentes cytokines

Le vecteur d'expression eucaryote pMLP10 a été décrit (Ballay et al., 1985). Un dérivé de ce vecteur (pMLP18) a été construit par insertion d'une séquence contenant différents sites uniques de restriction en aval du promoteur majeur tardif d'adénovirus. Ces sites permettant ainsi le clonage des différents gènes codant pour les cytokines choisies sous le contrôle du promoteur viral. En aval de cette séquence contenant ces sites uniques de restriction a été placée la séquence contenant le signal de polyadénylation du gène codant pour les antigènes précoces du virus SV40. Le fragment BgIII - HindIII d'Ad5 est cloné en aval. Cette séquence de 3 Kpb contient le gène codant pour la protéine IX qui est nécessaire pour l'encapsidation du génome viral au-delà de 97 % de sa taille normale et permet la recombinaison in vivo ultérieure. Les séquences codant pour les gènes des différentes cytokines sont isolées à partir de plasmides obtenus auprès de différentes équipes. Ces séquences, obtenues après clivage au moyen de différentes enzymes de restriction sont introduites dans le site de clonage multiple du vecteur d'expression décrit ci-dessus (pMLP-18). On obtient ainsi les différents plasmides dénommés pMLP-cytokine (IL-2, IL-4 etc...) qui sont utilisés pour l'obtention des virus recombinants comme décrit dans le paragraphe suivant.

c) Transfection d'ADN et isolement de virus recombinants

Les adénovirus recombinants défectifs Ad-cytokines ont été obtenus par recombinaison in vivo entre le fragment droit du génome viral clivé au

préalable par l'enzyme de restriction Cla I et la séquence homologue existant sur les plasmides pMLP-cytokine décrits ci-dessus. Le mélange du fragment du génome viral (2,6 m.u. - 100 m.u.) purifié après clivage et du plasmide linéarisé par l'enzyme de restriction Cla I ou Pvu I est transfecté dans les cellules 293 en utilisant la méthode de précipitation au phosphate de calcium (Graham et Van Der Eb, 1973). Des plages de cellules montrant un effet cytopathique ont été isolées 10 jours après et le virus a été amplifié en culture. L'ADN viral a été extrait par la procédure Hirt (Graham et al., 1977) et les virus recombinants ont été identifiés par cartographie avec des enzymes de restriction.

La figure 1 représente schématiquement une construction de ce type mettant en oeuvre une séquence d'insertion codant pour une interleukine (IL-2, IL-4, etc..).

Dans cette figure :

- "leaders" correspond à un leader tripartite
- "Del" correspond à une "délétion"
- Ad dl 324 correspond à un adénovirus pourvu des "délétions" sus-indiquées.

d) Expression des séquences codant pour une cytokine exprimée

Des lignées cellulaires Hela ou Vero sont infectées avec les virus recombinants défectifs obtenus. Les cellules effectivement transfectées peuvent être caractérisées essentiellement grâce à la détection de l'activité de la cytokine libérée dans leur milieu de culture. Dans le cas de IL-II des rendements pouvant atteindre de 1 à 2 μ g d'interleukine pour 10^6 cellules ont été observés.

Les cellules infectées par un recombinant Ad-cytokine sécrètent dans le milieu de culture des quantités variables de la cytokine. Il existe

différentes méthodes pour la détection et la quantification des cytokines produites.

1) Méthodes quantitatives :

- ELISA, en utilisant des anticorps spécifiques
- RIA (radioimmunoassay)
- Western blot

2) Méthodes qualitatives (ou biotests) : basées sur les propriétés biologiques des cytokines

Par exemple :

IL-2 : Test de prolifération des cellules CTL-L2 (les cellules CTL-L2 ne se multiplient et ne se maintiennent en culture qu'en présence d'IL-2 dans le milieu de culture)

IL-3 et GM-CSF : Test de prolifération des cellules TF-1

IL-4 : Test de prolifération des cellules CTL-L2 et induction de CD23 solubles par certaines cellules dont les lymphocytes.

INF- α : Test de cytotoxicité sur les cellules L92-9.

Test de neutralisation : L'effet des cytokines peut être bloqué en incubant les cellules cibles en présence de cellules d'anticorps spécifiques.

Quelques résultats obtenus avec le vecteur adénovirus portant le gène de l'IL-2(Ad-IL-2) sont exposés ci-après.

1) Les cellules infectées in vitro avec l'Ad-IL-2 sécrétant des quantités significatives d'IL-2 fonctionnelle.

2) L'injection directe du vecteur portant le gène de l'IL-2 dans des tumeurs déjà établies chez l'animal (le diamètre tumoral au moment de l'injection est entre 4 et 7 mm) induit la stimulation des systèmes immunitaires qui se traduit par une stabilisation de la taille de la tumeur, puis sa régression jusqu'à disparition complète dans 40 % à 50 % des cas.

12.

Ce résultat peut être amélioré en traitant les tumeurs dans une phase plus précoce de son développement ou en utilisant différents vecteurs à la fois par exemple association de Ad-IL-2 avec Ad-INF et/ou Ad-IL-4, Ad-GM-CSF, Ad-IL-3. Cette combinaison est à préciser selon le type de tumeur.

3) Les cellules tumorales infectées in vitro puis injectées à des animaux syngéniques ou même à des animaux immunodéficients (souris Nu/Nu) perdent leur pouvoir tumorigène (au moins jusqu'à 80 % des animaux rejettent les cellules tumorales ; en d'autres termes, les cellules tumorales ne prolifèrent plus chez 80 % des animaux immunodéficients injectés avec ces cellules.

4) Les animaux ayant rejeté une première injection de cellules tumorales infectées sont hautement immunisés et sont protégés contre des cellules tumorales parentales (non infectées) et injectées à différents temps et à différents endroits.

Co-injectées avec des cellules tumorales infectées in vitro, les cellules spléniques de ces animaux immunisés sont en plus capables de transférer l'immunité anti-tumorale à des animaux récipiends.

Il va de soi que les descriptions de constructions d'adénovirus défectifs recombinants ci-dessus envisagées n'ont aucun caractère restrictif. D'autres constructions peuvent être réalisées, notamment selon les variantes encore indiquées ci-après à titre d'exemples.

1) Echange des promoteurs

Le promoteur majeur tardif d'Adénovirus peut être substitué par d'autres promoteurs ubiquitaires mais d'origine exogène tels que :

- promoteur contenu dans le LTR (Long Terminal Repeat) de RSV (Rous Sarcome Virus)
- le promoteur du gène IE de CMV (cytomégalovirus)

- les promoteurs inductibles MMTV (Mouse Mammary Tumor Virus) ou métallothionine.

De même des promoteurs permettant une expression plus spécifique, restreinte aux cellules tumorales peuvent être utilisés comme par exemple :

- le promoteur du gène rep du parvovirus HI.

L'invention concerne encore un acide nucléique recombinant du type sus-indiqué, caractérisé en ce que la séquence génomique de l'adénovirus est dépourvue de sa région d'extrémité 5', en aval du promoteur précoce de la région E1A de l'adénovirus, et en ce que la séquence codant pour la cytokine est placée sous le contrôle de ce promoteur précoce. Cet acide nucléique recombinant peut également être mis en oeuvre dans les applications plus particulièrement mentionnées en rapport avec les ADNs recombinants dans lesquels la séquence codant pour la cytokine est placée sous le contrôle du promoteur majeur tardif de l'adénovirus.

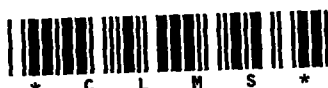
2) Expression simultanée de plusieurs gènes de cytokines

3 types de constructions sont décrites :

- les gènes de cytokines sont sous le contrôle de deux promoteurs soit identiques, soit différents (MLP et RSV par exemple) et situés à la suite l'un de l'autre.
- les gènes des cytokines sont sous le contrôle de promoteurs distincts et clonées dans des régions distinctes du virus.

REVENDICATIONS

1. Acide nucléique recombinant comportant, d'une part, une séquence génomique d'un adénovirus défectif en ce qu'elle est dépourvue des séquences nécessaires à sa réplication, mais comportant néanmoins celles des séquences qui dans ce génome sont le support de l'information génétique nécessaire à l'adénovirus correspondant pour pénétrer dans les cellules infectables par celui-ci, ainsi que l'ensemble des séquences essentielles nécessaires à l'encapsidation de cet adénovirus, et, d'autre part, un insérat contenant une séquence nucléique codant pour une cytokine, cet insérat étant sous le contrôle d'un promoteur présent ou préalablement inséré dans la susdite séquence génomique.
2. Acide nucléique recombinant selon la revendication 1 caractérisé en ce qu'il est dépourvu des transactivateurs E1A et E1B et, le cas échéant, de la région E3 de l'adénovirus.
3. Acide nucléique recombinant selon la revendication 1 caractérisé en ce que la séquence génomique de l'adénovirus est dépourvue de sa région d'extrémité 5', en aval du promoteur précoce de la région E1A de l'adénovirus, et en ce que la séquence codant pour la cytokine est placée sous le contrôle de ce promoteur précoce.
4. Acide nucléique recombinant selon la revendication 1 ou la revendication 2 caractérisé en ce que la séquence codant pour la cytokine est placée sous le contrôle d'un promoteur tardif de l'adénovirus.
5. Acide nucléique recombinant selon la revendication 1, caractérisé en ce que la séquence génomique de l'adénovirus est pourvue d'un promoteur



étranger au génome de l'adénovirus, et en ce que la séquence codant pour la cytokine est placée sous le contrôle de ce promoteur étranger.

6. Acide nucléique recombinant caractérisé, soit en ce que l'insérat contient des séquences codant pour plusieurs cytokines, soit en ce qu'il contient des insérats distincts respectivement placés sous le contrôle de promoteurs distincts, également distincts.

7. Adénovirus défectif caractérisé en ce qu'il contient l'acide nucléique recombinant selon l'une quelconque des revendications 1 à 6.

8. Culture de cellules, notamment d'origine humaine, caractérisée en ce qu'elles sont infectées par l'adénovirus selon la revendication 7.

9. Composition pharmaceutique contenant l'adénovirus recombinant selon la revendication 7 en association avec un véhicule pharmaceutiquement acceptable, notamment.

10. Utilisation de l'adénovirus recombinant selon la revendication 7 pour la préparation de médicaments antitumoraux, de préférence sous forme directement injectable dans une tumeur de l'hôte.

11. Composition pharmaceutique contenant des cellules selon la revendication 8, de préférence humaines, dans un état autorisant leur injection à l'homme.

12. Procédé de production d'adénovirus défectifs recombinants selon la revendication 7 caractérisé par la transformation de lignées cellulaires transformables d'eucaryotes supérieurs (notamment d'origine humaine ou animale) comportant elles-mêmes une séquence distincte de nucléotides apte à compléter la partie du génome de l'adénovirus dont celui-ci est dépourvu et qui serait essentielle à sa répllication, ladite séquence distincte étant de préférence incorporée au génome des cellules de ladite lignée cellulaire, et en ce que l'on récupère les



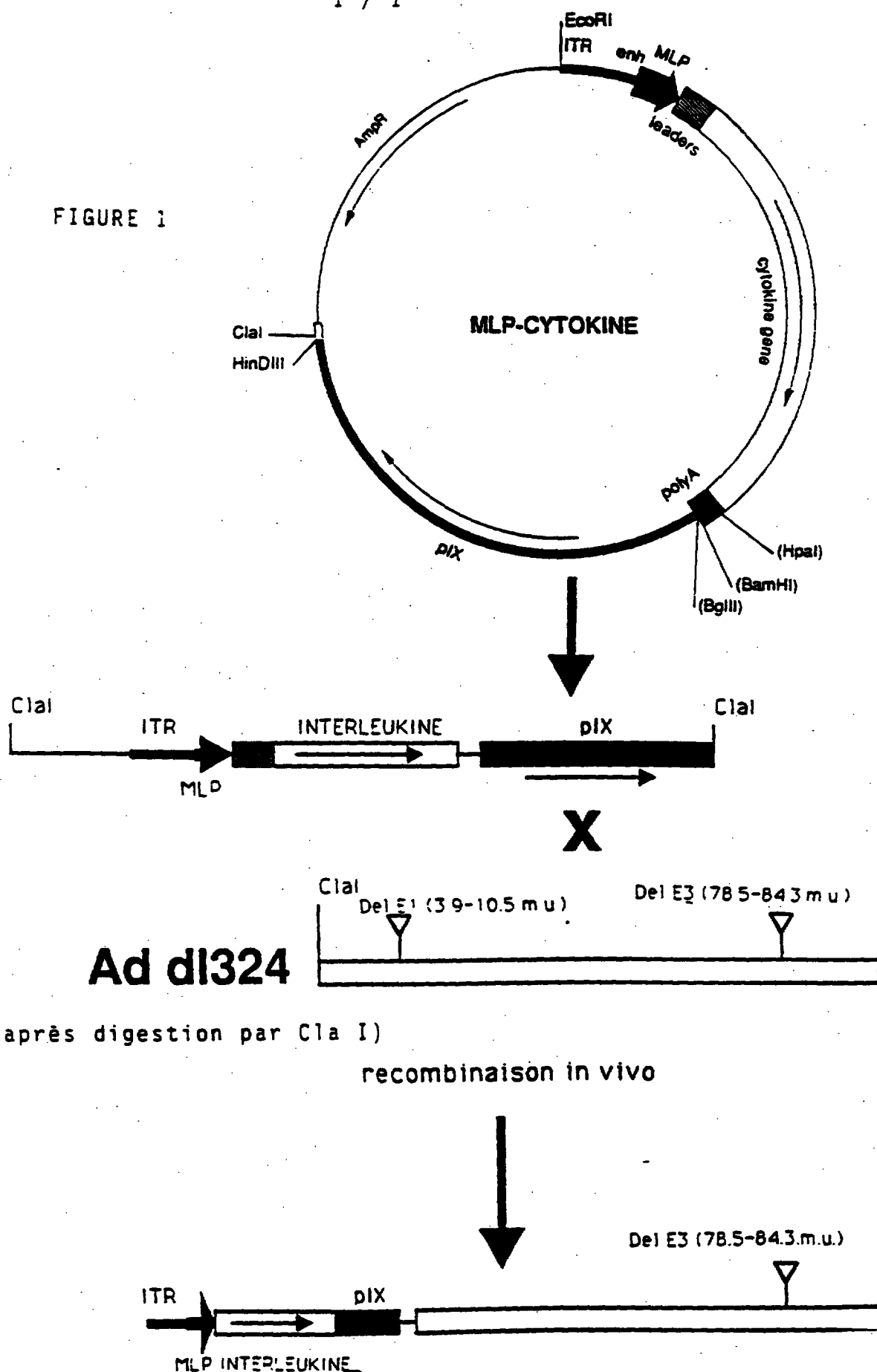
adénovirus recombinants défectifs produits à partir du milieu de culture des cellules desdites lignées cellulaires.

13. Procédé selon la revendication 12 caractérisé en ce que le génome d'adénovirus défectifs est dépourvu de sa région d'extrémité 5' et que la lignée cellulaire est une lignée de rein embryonnaire humain telle que la lignée 293, qui contient, intégré dans son génome, une région d'extrémité 5' du génome d'un adénovirus de type 5 (Ad5) et ayant une taille correspondante à approximativement 11 % de celle du génome entier de cet adénovirus.



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FIGURE 1



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**DEFECTIVE RECOMBINANT ADENOVIRUSES EXPRESSING
CYTOKINES FOR ANTITUMOR TREATMENT**

Cytokines are molecules (hormones) produced by cells following an antigenic stimulation or an activation
5 by other factors. The first cytokine which will has been produced is interleukin-1 (Il-1). It permits activation of the T cells which, in turn, start producing a whole battery of lymphokines, some of which are essential for the activation of the immune system and the defenses
10 against viral or parasitic infections.

For some years, cytokines have been used in anticancer immunotherapy. Nevertheless, when they are administered systemically, a number of problems arise. Il-2, for example, produces quite substantial side effects;
15 it is rapidly metabolized, so that high doses have to be administered repeatedly.

Better administration routes which would increase their efficacy while decreasing their adverse effects are hence being sought.

20 The subject of the invention is hence defective recombinant adenoviruses expressing one or more cytokines, as well as the use of these recombinant adenoviruses for making pharmaceutical compositions, in particular antitumor compositions, more especially compositions
25 tions which can be injected directly into solid-tumors of the host.

The subject of the present invention is defective recombinant adenoviruses, characterized in that they contain a defective, non-replicable adenovirus genome



into which one or more nucleic acid sequences coding for one more cytokines, in particular lymphokines, are inserted under the control of one or more promoters capable of being recognised by the polymerases of human
5 cells, more especially of human tumor cells or of cells infiltrating these tumors.

The invention relates more especially to recombinant nucleic acids capable of being used for the production of such defective recombinant adenoviruses.

10 Such a recombinant nucleic acid is characterized in that it contains, on the one hand a genomic sequence of an adenovirus which is defective in that it lacks the sequences needed for its replication, but which nevertheless contains those sequences which, in this genome, are
15 the carrier of the genetic information needed for the corresponding adenovirus to enter the cells which the latter is capable of infecting, as well as the set of essential sequences needed for encapsidation of this adenovirus, and on the other hand an insert containing a
20 nucleic acid sequence coding for a cytokine, this insert being under the control of a promoter present in or previously inserted into the abovementioned genomic sequence.

Adenoviruses, in particular type 2 or 5 adenoviruses capable of infecting humans (or human adenoviruses), or alternatively serotype 4 and 7 adenoviruses, represent especially preferred vectors in the context of
25 the present invention, on account, in particular, of the large size of the foreign DNA fragment which it is



possible to insert into the genome of these viruses.

Advantageously the abovementioned nucleic acid insertion sequence(s), coding for one or more predetermined cytokines, are contained in a defective adenovirus genome lacking the essential nucleotide sequences needed for replication of these adenoviruses, and more especially the transactivators E1A and E1B and, where appropriate, the E3 region of the adenovirus, or alternatively its E1 and E3 regions.

10 In other words, the invention turns to good account the capacity of these defective recombinant adenoviruses to allow the insertion sequence they contain to be expressed in the cells they invade even when, on account of their defective character, they do not multiply therein. In other words, the objective of the invention is to cause cytokines to be secreted actually within the cells of the tumour to be treated (tumor cells themselves and cells, in particular lymphocytes, which infiltrate these tumors) when these cells have been 15 infected with these defective adenoviruses, especially when the latter are injected directly into the tumor. The cytokines produced will thus activate first and foremost, in situ, the cytotoxic cells infiltrating the tumor and those present in proximity to the tumor.

25 Regarding the sequence for insertion into the defective recombinant adenovirus genome, this may be chosen from all those which express a cytokine capable of exerting either a direct antitumor effect, or an activating effect on immunocompetent cells of the body, or both



together.

Among these cytokines, the following may be mentioned as examples: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, α interferon, γ interferon, tumor necrosis factor
5 alpha (TNF α).

The same recombinant adenoviruses may also be used in the case of certain diseases in which there is an immune deficiency and in the case of certain parasitic or viral diseases, especially γ , α interferon and/or GM-
10 CSF), in particular by administration systemically or via cells, preferably human cells, taken in a state that allows them to be injected into humans, these cells having previously been infected with a recombinant defective adenovirus according to the invention.

15 The properties of some of these cytokines are recalled below.

Interleukin-1 (IL-1):

This is produced essentially by activated macrophages and monocytes. Its molecular weight is
20 approximately 17 kilodaltons. It displays several activities, including:

- a) a chemoattractive action on polymorphonuclear cells and macrophages (1, 2),
- b) an increase in the cytotoxic activity of
25 spontaneous cytotoxic (natural killer or Nk cells),
- c) an induction of fever following an infection,
- d) most especially, the activation of T cells for the production of other factors.



Interleukin-2 (IL-2), Interleukin-4 (IL-4) and
Interleukin-5 (IL-5):

These are produced by activated T lymphocytes. The action of these cytokines is restricted to cells of the immune system, they cause their multiplication and their activation: IL-2 and IL-4 have been tested in antitumor immunotherapy in mice and in man. In mice, they act synergistically and cause tumor regression.

Interleukin-6 (IL-6)

This is produced by many cell types including T lymphocytes, macrophages, fibroblasts, etc. It induces the final differentiation of B lymphocytes, which become antibody producers.

Tumor Necrosis Factor α (TNF α):

This is a factor produced by macrophages. It has a dual action: a direct action on tumor cells causing their lysis and an activation of the immune system.

The use of TNF α in man must be carried out cautiously since a large number of cells possess the receptor for it: which accounts for the importance of inducing its secretion only locally, actually within the tumor, to limit its adverse effects on the other cells of the host.

Interleukin-3 (IL-3), Interleukin-7 (IL-7) and Colony
stimulating Factor (CSF).

These are hematopoietic growth factors. They are produced essentially by lymphocytes, monocytes and macrophages. They act at different levels of hematopoiesis, that is to say of the different stages of



differentiation of marrow cells to blood cells. In addition, CSF exerts very substantial effects on the body's primary defences as regards the bacterial defences, luring the macrophages to the sites of infection and increasing their capacity for phagocytosis.

In combination with IL-2 and IL-4, GM-CSF proves to be an important antitumor factor.

γ Interferon (IFN- γ)

This is a factor produced by activated T cells; it is endowed with antiviral properties; it inhibits the multiplication of viruses and parasites and causes the lysis of infected cells and some tumor cells.

α Interferon (IFN- α)

Produced by T cells and monocytes, this displays an antiviral and lytic effect on infected cells. IFN- α has been used in immunotherapy against some types of cancer, including mesothelium,

Naturally, the invention is not limited, as regards the choice of insertion sequences which can be used in adenoviruses according to the invention, to those sequences which have been identified above. Nevertheless, the latter are illustrative of the palette of possibilities which are afforded to the therapist, who is responsible for making the choice of the most suitable defective recombinant adenovirus to be used in the light of the nature of the tumours to be combated.

The invention also relates to pharmaceutical compositions comprising one or more recombinant vectors as described above, in combination with a



pharmaceutically acceptable vehicle, especially sterile, isotonic compositions which can be injected directly into the tumors to be treated, or dry, in particular lyophilized, compositions which, by the addition of
5 sterilized water or of physiological saline as the case may be, enable solutions which can be injected directly into the tumors to be made up or reconstituted.

Direct injection of non-replicable, modified adenoviruses into the tumor affords the advantage, on the
10 one hand, of avoiding diffusion of the recombinant adenoviruses in the general circulation, with the consequent side effects liable to be exerted by the cytokines expressed in places other than on the sites where the manifestation of their action is sought, in this instance
15 the tumor cells themselves or the cells, in particular lymphocytes, which infiltrate them or which are present in their immediate proximity. Preferably, the injection is carried out at the very least in at least one site of the primary tumor.

20 Neither is the invention limited to administration of the recombinant adenoviruses containing the sequences coding for the cytokines of the kind in question directly in the tumors. Any other administration route permitting access of these recombinant adenoviruses
25 to the tumor to be treated may be envisaged. In particular, use may be made of cells which are compatible with the host, for example human fibroblasts, preferably ones previously removed from the host him- or herself.

The invention also relates to cell cultures, for



example cultured fibroblasts previously infected with recombinant nucleic acids, more especially the defective adenoviruses defined above. These infected cells, where appropriate attenuated or rendered immunologically inert, for example by irradiation, contribute to the eradication of installed tumors when they are injected systemically. This injection may be envisaged either alone, or in addition to injection directly into the tumor.

The subject of the invention is also a method for obtaining the recombinant adenoviruses described above, which comprises, after the actual step of construction of a vector by introduction of one or more insertion nucleic acid(s) into the genome of the initial defective adenovirus, a step of transformation of transformable cell lines of higher eukaryotes (in particular of human or animal origin) themselves containing a separate nucleotide sequence capable of complementing the portion(s) lacking in the genome of the defective adenovirus and without which replication of the latter is prevented, said separate sequence preferably being incorporated in the genome of the cells of said cell line.

As a preferred example of such cell lines, there may be mentioned line 293, a human embryonic kidney line which contains, integrated in its genome, the first eleven percent of the left-hand end of the genome of a type 5 adenovirus (Ad5). This fraction can then complement defective recombinant viruses which carry deletions of this region. Such a production method is described, more especially, in European Patent Application



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After transformation of these cell lines, the defective adenoviruses thus multiplied and produced are recovered from the culture medium of the cells of these
5 lines and purified.

Further details of the present invention will be given in the description which follows of the possibilities of construction of a recombinant vector adenovirus containing at least one sequence coding for a
10 cytokine, especially a lymphokine.

I. METHODS

A) Cells and viruses

Ad-5-transformed human embryonic kidney cell line 293 (Graham et al., 1977) was used for the transfection
15 of DNA as well as for the multiplication and titration of adenovirus (Ad). In effect, cell line 293 complements the functions of the genes for E1A and E1B functions and permits the replication of defective Ad recombinants. For the construction of the recombinant Ad, human Ad5-d1324,
20 carrying deletions in the E1 region (3.9-10.5 m.u.) and E3 region (78.5-84.3 m.u.), was used (Shenk and Williams, 1984). Cell lines 293, HeLa and Vero were maintained in an Eagle minimum essential culture medium with 10% of fetal calf serum.

25 b) Construction of plasmids permitting the expression of different cytokines

The eukaryotic expression vector pMLP10 has been described (Ballay et al., 1985). A derivative of this vector (pMLP18) was constructed by insertion of a



sequence containing different single restriction sites downstream of the adenovirus major late promoter. These sites thus permit cloning of the different genes coding for the chosen cytokines under the control of the viral promoter. Downstream of this sequence containing these single restriction sites, the sequence containing the polyadenylation signal of the gene coding for the early antigens of SV40 virus were placed. The BgIII HindIII fragment of Ad5 is cloned downstream. This 3-Kbp sequence contains the gene coding for protein IX which is necessary for encapsidation of the viral genome exceeding 97% of its normal size, and permits subsequent in vivo recombination. Sequences coding for the genes for the different cytokines are isolated from plasmids obtained from different teams. These sequences, obtained after cleavage by means of different restriction enzymes, are introduced into the multiple cloning site of the expression vector described above (pMLP-18). The different plasmids designated pMLP-cytokine (IL-2, IL-4, and the like), which are used for obtaining the recombinant viruses as described in the following section, are thereby obtained.

c) Transfection of DNA and isolation of recombinant viruses

The Ad-cytokine defective recombinant adenoviruses were obtained by in vivo recombination between the straight fragment of the viral genome previously cleaved with the restriction enzyme Cla I and the homologous sequence existing on the plasmids



pMLP-cytokine described above. The mixture of the fragment of the viral genome (2.6 m.u. - 100 m.u.), purified after cleavage, and of the plasmid linearized with the restriction enzyme Cla I or Pvu I is transfected into
5 293 cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). Cell plaques showing a cytopathic effect are isolated 10 days later and the virus was amplified in culture. The viral DNA was extracted by the Hirt procedure (Graham et al., 1977) and
10 the recombinant viruses were identified by mapping with restriction enzymes.

Figure 1 shows diagrammatically a construction of this type employing an insertion sequence coding for an interleukin (IL-2, IL-4, and the like).

15 In this figure:

- "leaders" corresponds to a tripartite leader
- "Del" corresponds to a "deletion"
- Ad dl 324 corresponds to an adenovirus provided with the abovementioned "deletions".

20 d) Expression of the sequences coding for an expressed cytokine

Hela or Vero cell lines are infected with the defective recombinant viruses obtained. Cells effectively transfected may be characterized essentially by
25 means of detection of the activity of the cytokine released into their culture medium. In the case of IL-IL-II yields capable of reaching from 1 to 2 µg of interleukin per 10⁶ cells have been observed.

Cells infected with an Ad-cytokine recombinant



secrete variable amounts of the cytokine into the culture medium. Different methods exist for the detection and quantification of the cytokines produced.

1) Quantitative methods:

- 5
- ELISA, using specific antibodies
 - RIA (radioimmunoassay)
 - Western blotting

2) Qualitative methods (or biotests): based on the biological properties of cytokines

10 For example:

IL-2: Test of proliferation of CTL-L2 cells (CTL-L2 cells multiply and are maintained in culture only in the presence of IL-2 in the culture medium)

15 IL-3 and GM-CSF: Test of proliferation of TF-1 cells

IL-4: Test of proliferation of CTL-L2 cells and induction of soluble CD23 with certain cells including lymphocytes.

INF- α : Cytotoxicity test on L92-9 cells.

20 Neutralization test: The effect of cytokines may be blocked by incubating the target cells in the presence of cells of specific antibodies.

Some results obtained with the adenovirus vector carrying the IL-2 gene (Ad-IL-2) are described below.

25 1) Cells infected in vitro with Ad-IL-2 secrete significant amounts of functional IL-2.

2) Direct injection of the vector carrying the IL-2 gene into tumors already established in the animal (the tumor diameter at the time of injection is between 4 and



7 mm) induces stimulation of the immune system which manifests itself in a stabilization of the size of the tumor, and then its regression to the point of complete disappearance in 40% to 50% of cases.

5 This result may be improved by treating the tumors in an earlier phase of its development or by using different vectors at the same time, for example combination of Ad-IL-2 with Ad-INF and/or Ad-IL-4, Ad-GM-CSF, Ad-IL-3. This combination has to be specified
10 according to the type of tumor.

3) Tumor cells infected in vitro and then injected into syngeneic animals or even immunodeficient animals (Nu/Nu mice) lose their tumorigenic power (at least up to 80% of the animals reject the tumor cells; in other words, the
15 tumor cells no longer proliferate in 80% of immunodeficient animals injected with these cells.

4) Animals which have rejected a first injection of infected tumor cells are highly immunized and are protected against parent (uninfected) tumor cells injected
20 at different times and at different places.

When coinjected with tumor cells infected in vitro, the spleen cells of these immunized animals are, furthermore, capable of transferring the antitumor immunity to recipient animals.

25 It is self-evident that the descriptions of constructions of recombinant defective adenoviruses envisaged above are in no way limiting in character. Other constructions may be produced, in particular

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according to the variants also mentioned below as examples.

1) Promoter exchange

5 The adenovirus major late promoter may be replaced by other promoters which are ubiquitous but of exogenous origin, such as:

- promoter contained in the LTR (long terminal repeat) of RSV (Rouse sarcoma virus)
- the promoter of the IE gene of CMV (cytomegalovirus)
- 10 - The MMTV (mouse mammary tumor virus) or metallothionine inducible promoters.

Similarly, promoters permitting a more specific expression restricted to tumor cells, may be used, such as, for example:

- 15 - the promoter of the rep gene of parvovirus HI.

The invention also relates to a recombinant nucleic acid of the abovementioned type, characterized in that the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus, and in that the sequence coding for the cytokine is placed under the control of this
20 early promoter. This recombinant nucleic acid may also be employed in applications mentioned more especially in connection with recombinant DNAs in which the sequence
25 coding for the cytokine is placed under the control of the adenovirus major late promoter.

2) Simultaneous expression of several cytokine genes

3 types of constructions are described:

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- the cytokine genes are under the control of two promoters which are either identical or different (MLP and RSV, for example) and are located following one another.

- 5 - the cytokine genes are under the control of separate promoters cloned into separate regions of the virus.

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CLAIMS

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1. Recombinant nucleic acid containing, on the one hand a genomic sequence of an adenovirus which is defective in that it lacks the sequences needed for its replication, but which nevertheless contains those sequences which, in this genome, are the carrier of the genetic information needed for the corresponding adenovirus to enter the cells which the latter is capable of infecting, as well as the set of essential sequences needed for encapsidation of this adenovirus, and on the other hand an insert containing a nucleic acid sequence coding for a cytokine, this insert being under the control of a promoter present in or previously inserted into the abovementioned genomic sequence.
2. Recombinant nucleic acid according to claim 1, characterized in that it lacks the transactivators E1A and E1B and, where appropriate, the E3 region of the adenovirus.
3. Recombinant nucleic acid according to claim 1, characterized in that the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus, and in that sequence coding for the cytokine is placed under the control of this early promoter.
4. Recombinant nucleic acid according to claim 1 or claim 2, characterized in that the sequence coding for the cytokine is placed under the control of an adenovirus late promoter.
5. Recombinant nucleic acid according to claim 1,



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- characterized in that the genomic sequence of the adenovirus is provided with a promoter foreign to the adenovirus genome, and in that the sequence coding for the cytokine is placed under the control of this foreign promoter.
- 5
6. Recombinant nucleic acid, characterized, either in that the insert contains sequences coding for several cytokines, or in that it contains separate inserts placed, respectively, under the control of separate promoters, which are also separate.
- 10
7. Defective adenovirus, characterized in that it contains recombinant nucleic acid according to any one of claims 1 to 6.
8. Culture of cells, in particular of human origin, characterized in that they are infected with the adenovirus according to claim 7.
- 15
9. Pharmaceutical composition containing the recombinant adenovirus according to claim 7, in combination with a pharmaceutically acceptable vehicle, in particular.
- 20
10. Use of the recombinant adenovirus according to claim 7 for the preparation of antitumor drugs, preferably in a form which can be injected directly into a tumor of the host.
- 25
11. Pharmaceutical composition containing cells according to claim 8, preferably human cells, in a state allowing them to be injected into humans.
12. Method for the production of recombinant defective adenoviruses according to claim 7,



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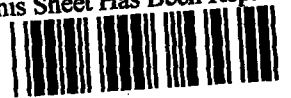


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characterized by the transformation of transformable cell lines of higher eukaryotes (in particular of human or animal origin) themselves containing a separate nucleotide sequence capable of complementing the portion
5 of the adenovirus genome which is lacking in the adenovirus and which would be essential for its replication, said separate sequence preferably being incorporated into the genome of the cells of said cell line, and in that the defective recombinant adenoviruses produced
10 are recovered from the culture medium of the cells of said cell lines.

13. Method according to claim 12, characterized in that the genome of the defective adenoviruses lacks its 5' end region, and in that the cell line is a human
15 embryonic kidney line such as line 293, which contains, integrated in its genome, a 5' end region of the genome of a type 5 adenovirus (Ad5) and having a size corresponding to approximately 11% of that of the whole genome of this adenovirus.

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ABSTRACT

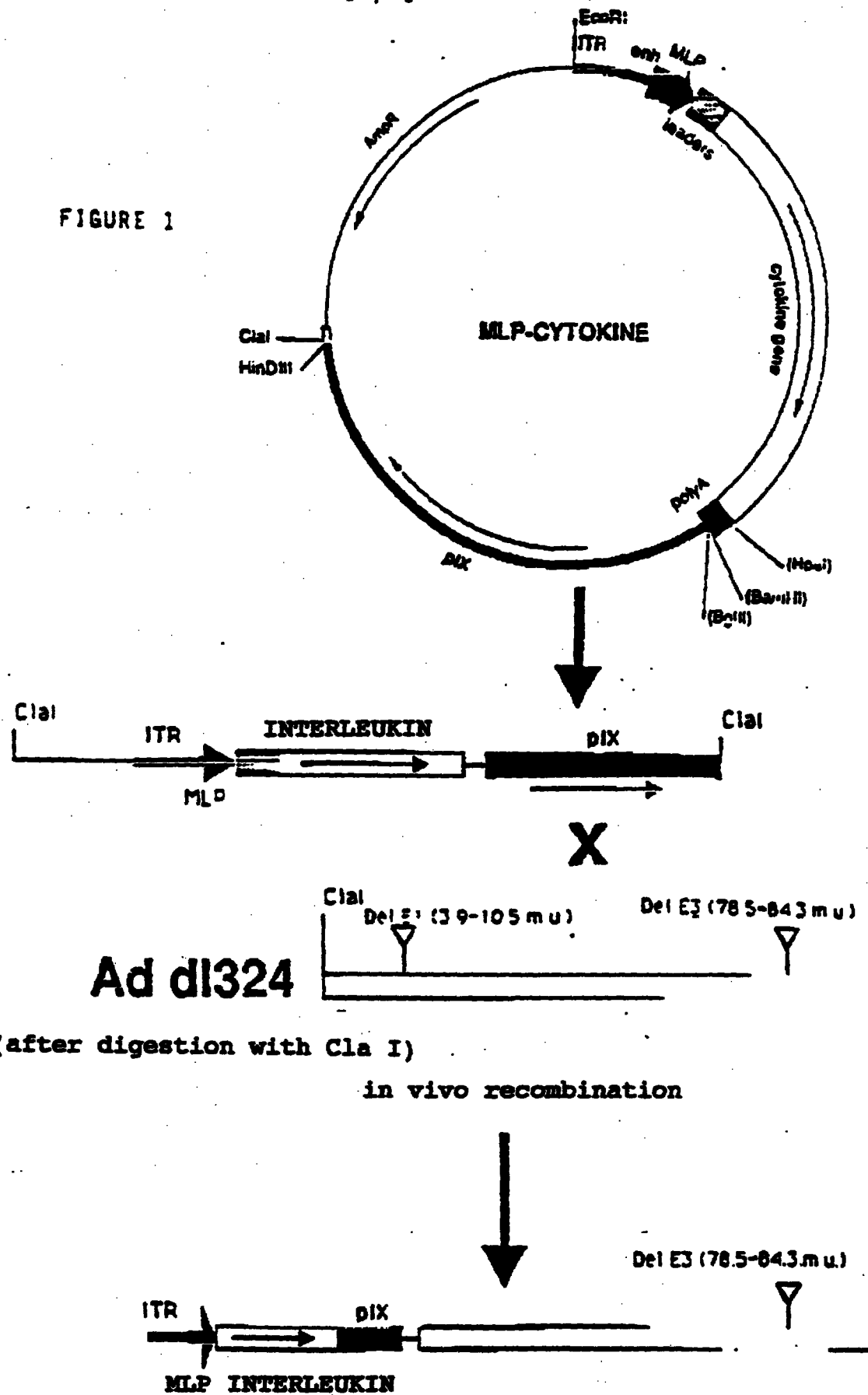
**DEFECTIVE RECOMBINANT ADENOVIRUSES
EXPRESSING CYTOKINES FOR ANTITUMOR TREATMENT**

Recombinant nucleic acid for use in the production of a defective adenovirus containing an inserted sequence coding for a cytokine under the control of a promotor in the genomic sequence of the recombinant adenovirus. Said recombinant adenovirus is useful in the preparation of anti-tumoral drugs capable of being directly injected into the tumor of the host.

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FIGURE 1



DEFECTIVE RECOMBINANT ADENOVIRUSES EXPRESSING

CYTOKINES FOR ANTITUMOR TREATMENT

Cytokines are molecules (hormones) produced by cells following an antigenic stimulation or an activation by other factors. The first cytokine which will has been produced is interleukin-1 (Il-1). It permits activation of the T cells which, in turn, start producing a whole battery of lymphokines, some of which are essential for the activation of the immune system and the defenses against viral or parasitic infections.

For some years, cytokines have been used in anticancer immunotherapy. Nevertheless, when they are administered systemically, a number of problems arise. Il-2, for example, produces quite substantial side effects;

it is rapidly metabolized, so that high doses have to be administered repeatedly.

Better administration routes which would increase their efficacy while decreasing their adverse effects are hence being sought.

The subject of the invention is hence defective recombinant adenoviruses expressing one or more cytokines, as well as the use of these recombinant adenoviruses for making pharmaceutical compositions, in particular antitumor compositions, more especially compositions which can be injected directly into solid tumors of the host.

The subject of the present invention is defective recombinant adenoviruses, characterized in that they contain a defective, non-replicable adenovirus genome

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into which one or more nucleic acid sequences coding for one more cytokines, in particular lymphokines, are inserted under the control of one or more promoters capable of being recognised by the polymerases of human cells, more especially of human tumor cells or of cells infiltrating these tumors.

The invention relates more especially to recombinant nucleic acids capable of being used for the production of such defective recombinant adenoviruses.

Such a recombinant nucleic acid is characterized in that it contains, on the one hand a genomic sequence of an adenovirus which is defective in that it lacks the sequences needed for its replication, but which nevertheless contains those sequences which, in this genome, are the carrier of the genetic information needed for the corresponding adenovirus to enter the cells which the latter is capable of infecting, as well as the set of essential sequences needed for encapsidation of this adenovirus, and on the other hand an insert containing a nucleic acid sequence coding for a cytokine, this insert being under the control of a promoter present in or previously inserted into the abovementioned genomic sequence.

Adenoviruses, in particular type 2 or 5 adenoviruses capable of infecting humans (or human adenoviruses), or alternatively serotype 4 and 7 adenoviruses, represent especially preferred vectors in the context of the present invention, on account, in particular, of the large size of the foreign DNA fragment which it is

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possible to insert into the genome of these viruses.

Advantageously the abovementioned nucleic acid insertion sequence(s), coding for one or more predetermined cytokines, are contained in a defective adenovirus genome lacking the essential nucleotide sequences needed for replication of these adenoviruses, and more especially the transactivators E1A and E1B and, where appropriate, the E3 region of the adenovirus, or alternatively its E1 and E3 regions.

In other words, the invention turns to good account the capacity of these defective recombinant adenoviruses to allow the insertion sequence they contain to be expressed in the cells they invade even when, on account of their defective character, they do not multiply therein. In other words, the objective of the invention is to cause cytokines to be secreted actually within the cells of the tumour to be treated (tumor cells themselves and cells, in particular lymphocytes, which infiltrate these tumors) when these cells have been infected with these defective adenoviruses, especially when the latter are injected directly into the tumor. The cytokines produced will thus activate first and foremost, in situ, the cytotoxic cells infiltrating the tumor and those present in proximity to the tumor.

Regarding the sequence for insertion into the defective recombinant adenovirus genome, this may be chosen from all those which express a cytokine capable of exerting either a direct antitumor effect, or an activating effect on immunocompetent cells of the body, or both

together.

Among these cytokines, the following may be mentioned as examples: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, α interferon, γ interferon, tumor necrosis factor alpha (TNF α).

The same recombinant adenoviruses may also be used in the case of certain diseases in which there is an immune deficiency and in the case of certain parasitic or viral diseases, especially γ , α interferon and/or GM-CSF), in particular by administration systemically or via cells, preferably human cells, taken in a state that allows them to be injected into humans, these cells having previously been infected with a recombinant defective adenovirus according to the invention.

The properties of some of these cytokines are recalled below.

Interleukin-1 (IL-1):

This is produced essentially by activated macrophages and monocytes. Its molecular weight is approximately 17 kilodaltons. It displays several activities, including:

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b) an increase in the cytotoxic activity of spontaneous cytotoxic (natural killer or Nk cells),

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Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Interleukin-5 (IL-5):

These are produced by activated T lymphocytes. The action of these cytokines is restricted to cells of the immune system, they cause their multiplication and their activation: IL-2 and IL-4 have been tested in antitumor immunotherapy in mice and in man. In mice, they act synergistically and cause tumor regression.

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In combination with IL-2 and IL-4, GM-CSF proves to be an important antitumor factor.

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Naturally, the invention is not limited, as regards the choice of insertion sequences which can be used in adenoviruses according to the invention, to those sequences which have been identified above. Nevertheless, the latter are illustrative of the palette of possibilities which are afforded to the therapist, who is responsible for making the choice of the most suitable defective recombinant adenovirus to be used in the light of the nature of the tumours to be combated.

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Neither is the invention limited to administration of the recombinant adenoviruses containing the sequences coding for the cytokines of the kind in question directly in the tumors. Any other administration route permitting access of these recombinant adenoviruses to the tumor to be treated may be envisaged. In particular, use may be made of cells which are compatible with the host, for example human fibroblasts, preferably ones previously removed from the host him- or herself.

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Further details of the present invention will be given in the description which follows of the possibilities of construction of a recombinant vector adenovirus containing at least one sequence coding for a cytokine, especially a lymphokine.

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pMLP-cytokine described above. The mixture of the fragment of the viral genome (2.6 m.u. - 100 m.u.), purified after cleavage, and of the plasmid linearized with the restriction enzyme Cla I or Pvu I is transfected into 293 cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). Cell plaques showing a cytopathic effect are isolated 10 days later and the virus was amplified in culture. The viral DNA was extracted by the Hirt procedure (Graham et al., 1977) and the recombinant viruses were identified by mapping with restriction enzymes.

Figure 1 shows diagrammatically a construction of this type employing an insertion sequence coding for an interleukin (IL-2, IL-4, and the like).

In this figure:

- "leaders" corresponds to a tripartite leader
- "Del" corresponds to a "deletion"
- Ad dl 324 corresponds to an adenovirus provided with the abovementioned "deletions".

d) Expression of the sequences coding for an expressed cytokine

Hela or Vero cell lines are infected with the defective recombinant viruses obtained. Cells effectively transfected may be characterized essentially by means of detection of the activity of the cytokine released into their culture medium. In the case of IL-IL-II yields capable of reaching from 1 to 2 μ g of interleukin per 10^6 cells have been observed.

Cells infected with an Ad-cytokine recombinant

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secrete variable amounts of the cytokine into the culture medium. Different methods exist for the detection and quantification of the cytokines produced.

1) Quantitative methods:

- ELISA, using specific antibodies
- RIA (radioimmunoassay)
- Western blotting

2) Qualitative methods (or biotests): based on the biological properties of cytokines

For example:

IL-2: Test of proliferation of CTL-L2 cells (CTL-L2 cells multiply and are maintained in culture only in the presence of IL-2 in the culture medium)

IL-3 and GM-CSF: Test of proliferation of TF-1 cells

IL-4: Test of proliferation of CTL-L2 cells and induction of soluble CD23 with certain cells including lymphocytes.

INF- α : Cytotoxicity test on L92-9 cells.

Neutralization test: The effect of cytokines may be blocked by incubating the target cells in the presence of cells of specific antibodies.

Some results obtained with the adenovirus vector carrying the IL-2 gene (Ad-IL-2) are described below.

1) Cells infected in vitro with Ad-IL-2 secrete significant amounts of functional IL-2.

2) Direct injection of the vector carrying the IL-2 gene into tumors already established in the animal (the tumor diameter at the time of injection is between 4 and

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7 mm) induces stimulation of the immune system which manifests itself in a stabilization of the size of the tumor, and then its regression to the point of complete disappearance in 40% to 50% of cases.

This result may be improved by treating the tumors in an earlier phase of its development or by using different vectors at the same time, for example combination of Ad-IL-2 with Ad-INF and/or Ad-IL-4, Ad-GM-CSF, Ad-IL-3. This combination has to be specified according to the type of tumor.

3) Tumor cells infected in vitro and then injected into syngeneic animals or even immunodeficient animals (Nu/Nu mice) lose their tumorigenic power (at least up to 80% of the animals reject the tumor cells; in other words, the tumor cells no longer proliferate in 80% of immunodeficient animals injected with these cells.

4) Animals which have rejected a first injection of infected tumor cells are highly immunized and are protected against parent (uninfected) tumor cells injected at different times and at different places.

When coinjected with tumor cells infected in vitro, the spleen cells of these immunized animals are, furthermore, capable of transferring the antitumor immunity to recipient animals.

It is self-evident that the descriptions of constructions of recombinant defective adenoviruses envisaged above are in no way limiting in character. Other constructions may be produced, in particular

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according to the variants also mentioned below as examples.

1) Promoter exchange

The adenovirus major late promoter may be replaced by other promoters which are ubiquitous but of exogenous origin, such as:

- promoter contained in the LTR (long terminal repeat) of RSV (Rouse sarcoma virus)
- the promoter of the IE gene of CMV (cytomegalovirus)
- The MMTV (mouse mammary tumor virus) or metallothionein inducible promoters.

Similarly, promoters permitting a more specific expression restricted to tumor cells, may be used, such as, for example:

- the promoter of the rep gene of parvovirus HI.

The invention also relates to a recombinant nucleic acid of the abovementioned type, characterized in that the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus, and in that the sequence coding for the cytokine is placed under the control of this early promoter. This recombinant nucleic acid may also be employed in applications mentioned more especially in connection with recombinant DNAs in which the sequence coding for the cytokine is placed under the control of the adenovirus major late promoter.

2) Simultaneous expression of several cytokine genes

3 types of constructions are described:

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- the cytokine genes are under the control of two promoters which are either identical or different (MLP and RSV, for example) and are located following one another.

- the cytokine genes are under the control of separate promoters cloned into separate regions of the virus.

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CLAIMS

1. Recombinant nucleic acid containing, on the one hand a genomic sequence of an adenovirus which is defective in that it lacks the sequences needed for its replication, but which nevertheless contains those sequences which, in this genome, are the carrier of the genetic information needed for the corresponding adenovirus to enter the cells which the latter is capable of infecting, as well as the set of essential sequences needed for encapsidation of this adenovirus, and on the other hand an insert containing a nucleic acid sequence coding for a cytokine, this insert being under the control of a promoter present in or previously inserted into the abovementioned genomic sequence.

2. Recombinant nucleic acid according to claim 1, characterized in that it lacks the transactivators E1A and E1B and, where appropriate, the E3 region of the adenovirus.

3. Recombinant nucleic acid according to claim 1, characterized in that the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus, and in that sequence coding for the cytokine is placed under the control of this early promoter.

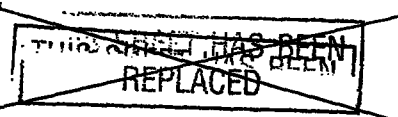
4. Recombinant nucleic acid according to claim 1 or claim 2, characterized in that the sequence coding for the cytokine is placed under the control of an adenovirus late promoter.

5. Recombinant nucleic acid according to claim 1,

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characterized in that the genomic sequence of the adenovirus is provided with a promoter foreign to the adenovirus genome, and in that the sequence coding for the cytokine is placed under the control of this foreign promoter.

6. Recombinant nucleic acid, characterized, either in that the insert contains sequences coding for several cytokines, or in that it contains separate inserts placed, respectively, under the control of separate promoters, which are also separate.

7. Defective adenovirus, characterized in that it contains recombinant nucleic acid according to any one of claims 1 to 6.

8. Culture of cells, in particular of human origin, characterized in that they are infected with the adenovirus according to claim 7.

9. Pharmaceutical composition containing the recombinant adenovirus according to claim 7, in combination with a pharmaceutically acceptable vehicle, in particular.

10. Use of the recombinant adenovirus according to claim 7 for the preparation of antitumor drugs, preferably in a form which can be injected directly into a tumor of the host.

11. Pharmaceutical composition containing cells according to claim 8, preferably human cells, in a state allowing them to be injected into humans.

12. Method for the production of recombinant defective adenoviruses according to claim 7.

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characterized by the transformation of transformable cell lines of higher eukaryotes (in particular of human or animal origin) themselves containing a separate nucleotide sequence capable of complementing the portion of the adenovirus genome which is lacking in the adenovirus and which would be essential for its replication, said separate sequence preferably being incorporated into the genome of the cells of said cell line, and in that the defective recombinant adenoviruses produced are recovered from the culture medium of the cells of said cell lines.

13. Method according to claim 12, characterized in that the genome of the defective adenoviruses lacks its 5' end region, and in that the cell line is a human embryonic kidney line such as line 293, which contains, integrated in its genome, a 5' end region of the genome of a type 5 adenovirus (Ad5) and having a size corresponding to approximately 11% of that of the whole genome of this adenovirus.



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ABSTRACT

**DEFECTIVE RECOMBINANT ADENOVIRUSES
EXPRESSING CYTOKINES FOR ANTITUMOR TREATMENT**

Recombinant nucleic acid for use in the production of a defective adenovirus containing an inserted sequence coding for a cytokine under the control of a promotor in the genomic sequence of the recombinant adenovirus. Said recombinant adenovirus is useful in the preparation of anti-tumoral drugs capable of being directly injected into the tumor of the host.

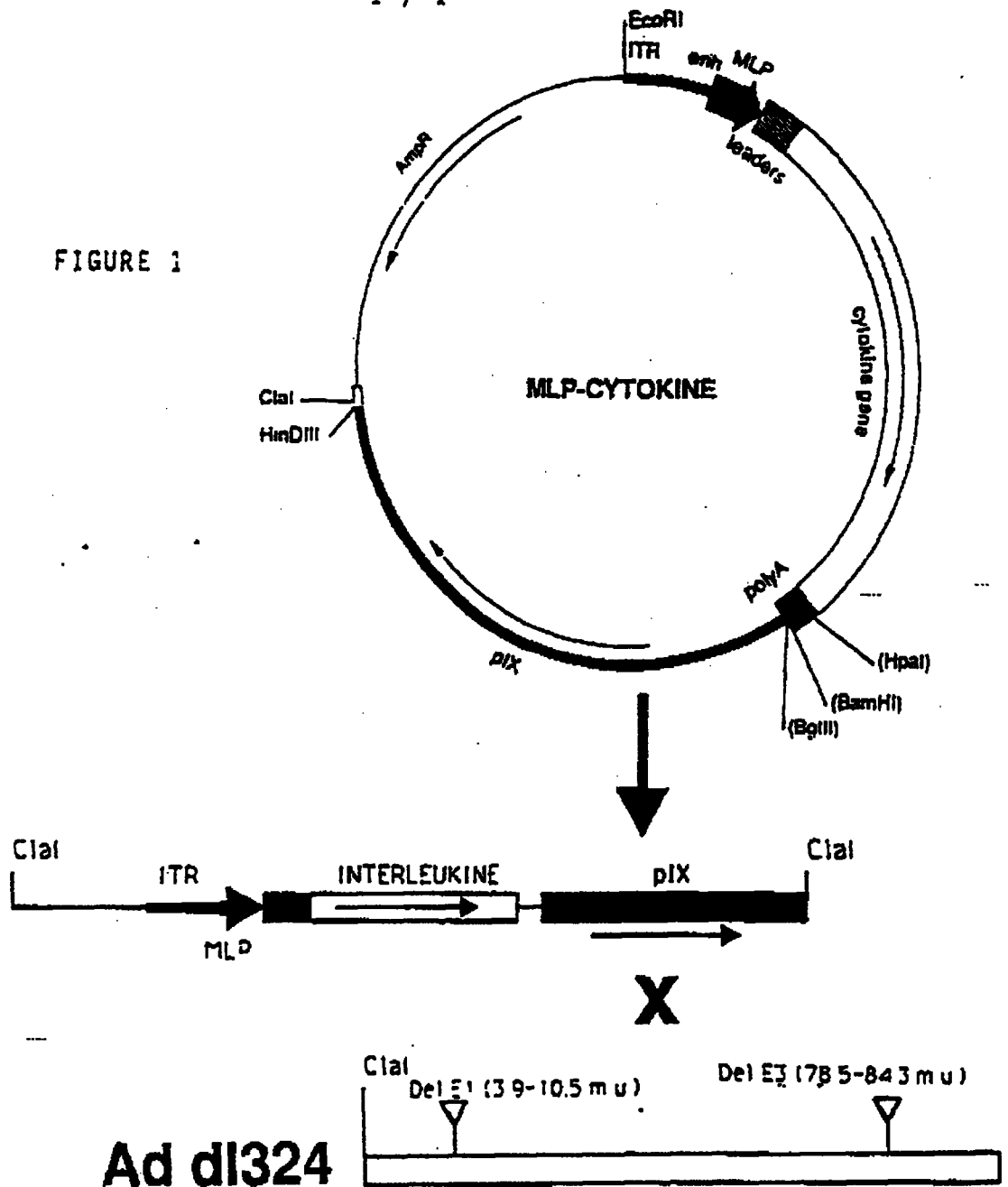
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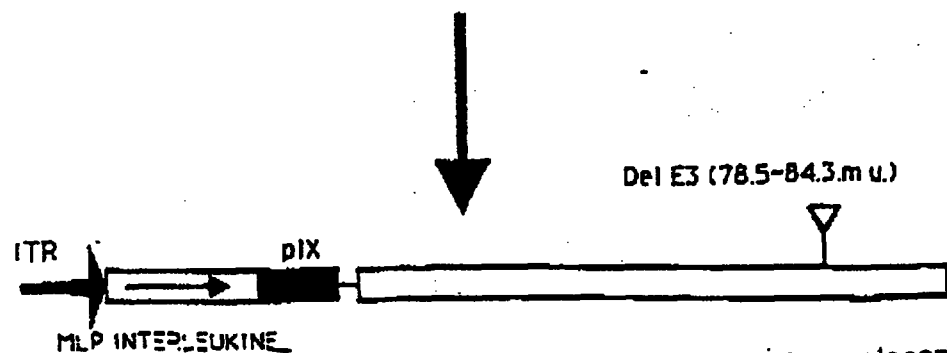
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FIGURE 1



après digestion par **Cla I**)

recombinaison in vivo



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ABSTRACT

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28 FEB 1997

**DEFECTIVE RECOMBINANT ADENOVIRUSES
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Recombinant nucleic acid for use in the production of a defective adenovirus containing an inserted sequence coding for a cytokine under the control of a promotor in the genomic sequence of the recombinant adenovirus. Said recombinant adenovirus is useful in the preparation of anti-tumoral drugs capable of being directly injected into the tumor of the host.

Figure 1

CLAIMS

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1 An adenoviral vector comprising a genomic sequence of an adenovirus wherein said genomic sequence:

(a) is defective in that said adenovirus lacks the sequences needed for its replication, but which contains those sequences which carry genetic information needed for the corresponding adenovirus to enter the cells which adenovirus is capable of infecting;

(b) comprises a set of essential sequences needed for encapsidation of said adenovirus; and

(c) comprises an insert containing a nucleic acid sequence coding for a cytokine wherein said insert is under the control of an endogenous or heterologous promoter, wherein said adenoviral vector is used as an antitumor drug.

2. The adenoviral vector according to Claim 1, wherein said vector lacks the transactivators E1A and E1B and the E3 region of the adenovirus.

3. The adenoviral vector according to Claim 1, wherein the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus, and wherein the nucleic acid sequence coding for the cytokine is placed under the control of the early promoter.



4. The adenoviral vector according to Claim 1 or Claim 2, wherein the nucleic acid coding for the cytokine is placed under the control of an adenovirus late promoter.
5. The adenoviral vector according to Claim 1, wherein the genomic sequences of the adenovirus has a heterologous promoter and wherein said nucleic acid sequence coding for the cytokine is placed under the control of said foreign heterologous promoter.
6. A defective adenoviral vector comprising a nucleic acid insert containing sequences coding for several cytokines, or containing separate nucleic acid inserts coding for different cytokines wherein said nucleic acids are placed under the control of separate promoters and wherein said adenoviral vector is used as an antitumor drug.
7. A defective adenovirus comprising the defective adenoviral vector according to any one of Claims 1 to 6.
8. A cell culture transformed with the defective adenoviral vector according to Claim 1 or infected with the defective adenoviral vector according to Claim 7.
9. A pharmaceutical composition comprising the defective adenovirus according to Claim 7, in combination with a pharmaceutically acceptable vehicle.
10. The pharmaceutical composition according to Claim 9, wherein said defective adenoviral vector lacks the transactivators E1A and E1B and where appropriate the E3 region of the adenovirus.



11. The pharmaceutical composition of Claim 10, wherein the genomic sequence of the adenovirus lacks its 5' end region downstream of the early promoter of the E1A region of the adenovirus and the nucleic acid sequence coding for the cytokine is placed under the control of this early promoter.
12. The pharmaceutical composition of Claim 10, wherein the nucleic acid sequence coding for the cytokine is placed under the control of an adenovirus late promoter.
13. The pharmaceutical composition according to Claim 10, wherein the genomic sequence of the adenovirus has a heterologous promoter.
14. The pharmaceutical composition of Claim 9, wherein the nucleic acid insert contains sequences coding for several cytokines or said nucleic acid insert contains separate inserts coding for different cytokines wherein said separate inserts are placed under the control of separate promoters.
15. The pharmaceutical composition of Claim 9, which can be injected directly into a tumor of the host.
16. A method for the treatment of tumors in a patient carrying a solid tumor which method comprises injecting an effective amount of the pharmaceutical composition of Claim 9 into said solid tumor.
17. The cell culture of Claim 8, which is a culture of human cells injectable into humans.
18. A method for activating an immune system and defenses against viral or parasitic infections or tumor development in a patient which comprises



administering systematically to said patient an effective amount of a cell culture of Claim 8.

19. A method for producing a recombinant defective adenovirus according to Claim 7, comprising the steps of:

(a) transforming a higher eukaryote cell line whose cells contain a separate nucleotide sequence capable of complementing the portion of the adenovirus genome which is lacking in the adenovirus and which is essential for its replication, said sequence being incorporated into the genome of the cells of said cell line; and

(b) recovering the defective recombinant adenoviruses produced from the culture medium of the cells of said cell line.

